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Molecular phylogeny of *Lytorhynchus diadema* (Reptilia, Colubridae) populations in Saudi Arabia

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Abstract

This study presents the molecular phylogenetic relationships among *Lytorhynchus diadema* (Duméril, Bibron & Duméril, 1854) populations in Saudi Arabia relative to populations from Africa and Asia. This phylogenetic analysis was based on mitochondrial 16S and 12S rRNA partial gene fragments using Neighbor-joining, Maximum Parsimony, and Bayesian methods. The results strongly support the monophyly of *Lytorhynchus* based on two concatenated genes and the 12S rRNA gene separately. Also, a significant separation is observed between the Arabian samples from Saudi Arabia, Yemen, and Oman, and the African populations from Egypt, Tunisia, and Morocco.

Key Words

Colubridae, *Lytorhynchus*, mtDNA, phylogeny, Saudi Arabia

Introduction

The genus *Lytorhynchus* Peters, 1863 contains six described species encompassing a vast geographical distribution and range of habitats (Leviton and Anderson 1970; Leviton 1977; Torki 2017; Uetz et al. 2021). The Diademed sand Snake, *Lytorhynchus diadema* (Duméril, Bibron & Duméril, 1854), is a non-venomous colubrid snake (Anderson 1898). It is a nocturnal species that inhabit vegetated sand dunes, gravel plains, and salt flats called "sabkhah" (Gasperetti 1988; Amr and Disi 2011; Ibrahim 2013). The range of *L. diadema* extends from Morocco in the west across North Africa towards Arabia and Iran (Gasperetti 1988; Al-Sadoon 1989; Schätti and Gasperetti 1994; Baha El Din 2006; Sindaco et al. 2013; Al-Sadoon et al. 2017; Alshammari et al. 2017). Although it was previously suggested that *L. gaddi* Nikolsky, 1907 was a subspecies of *L. diadema* (Leviton et al. 1992), Schätti and Gasperetti (1994) defined it as separate species, therefore increasing the number of species in the genus to seven.

To assess the geographic variation and genetic diversity within the range of *L. diadema*, samples from Saudi Arabia were collected, sequenced, and compared with samples from across Arabia and North Africa. The partial mitochondrial 16S and 12S rRNA sequenced were also compared to two congeners of *L. maynardi* (Alcock and Finn 1896) and *L. gaddi*.

Materials and methods

Snakes were collected from the Ha'il and Ta'if provinces of Saudi Arabia (Fig. 1, Table 1) according to the ethical

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Table 1. A list of *Lytorhynchus* samples collected from Saudi Arabia used in this study and GenBank accession numbers of 16S rRNA and 12S rRNA previously used in phylogenetic studies with the relative sources.

Species	Site	Country	Latitude, Longitude	16S	12S	Reference
L. diadema	Ta'if	Saudi Arabia	21.388, 40.531	HQ267793	HQ658442	This study
	Ha'il		27.528, 41.739	HQ267794	-	
	Ha'il		27.528, 41.739	HQ267795	HQ658430	
	Ha'il/Al-Fatkha		27.456, 41.293	HQ267796	HQ658425	
	Ha'il		27.528, 41.739	-	HQ658422	
	North Sinai	Egypt	31.045, 33.416	KX909295	KX909261	Tamar et al. 2016
	-	Egypt	-	AY643351	AY643309	Carranza et al. 2004
	Djébil	Tunisia	35.762, 9.647	AY188064	-	Nagy et al. 2003
	-	Morocco	-	KX909294	-	Tamar et al. 2016
	Wal Wafi	Oman	22.308, 59.221	KX909293	KX909259	
	Jabal Mafluq	Yemen	16.629, 43.984	-	AY647229	Schätti and Monsch 2004
L. gaddi	-	Iran	49.236, 31.273	KX909296	KX909262	Tamar et al. 2016
L. maynardi	Bampur	Iran	27.253, 60.409	KX909316	-	
	Hatay	Pakistan	29.389, 65.684	-	KX909286	
	Hatay	Pakistan	29.389, 65.684	KX909317	KX909285	

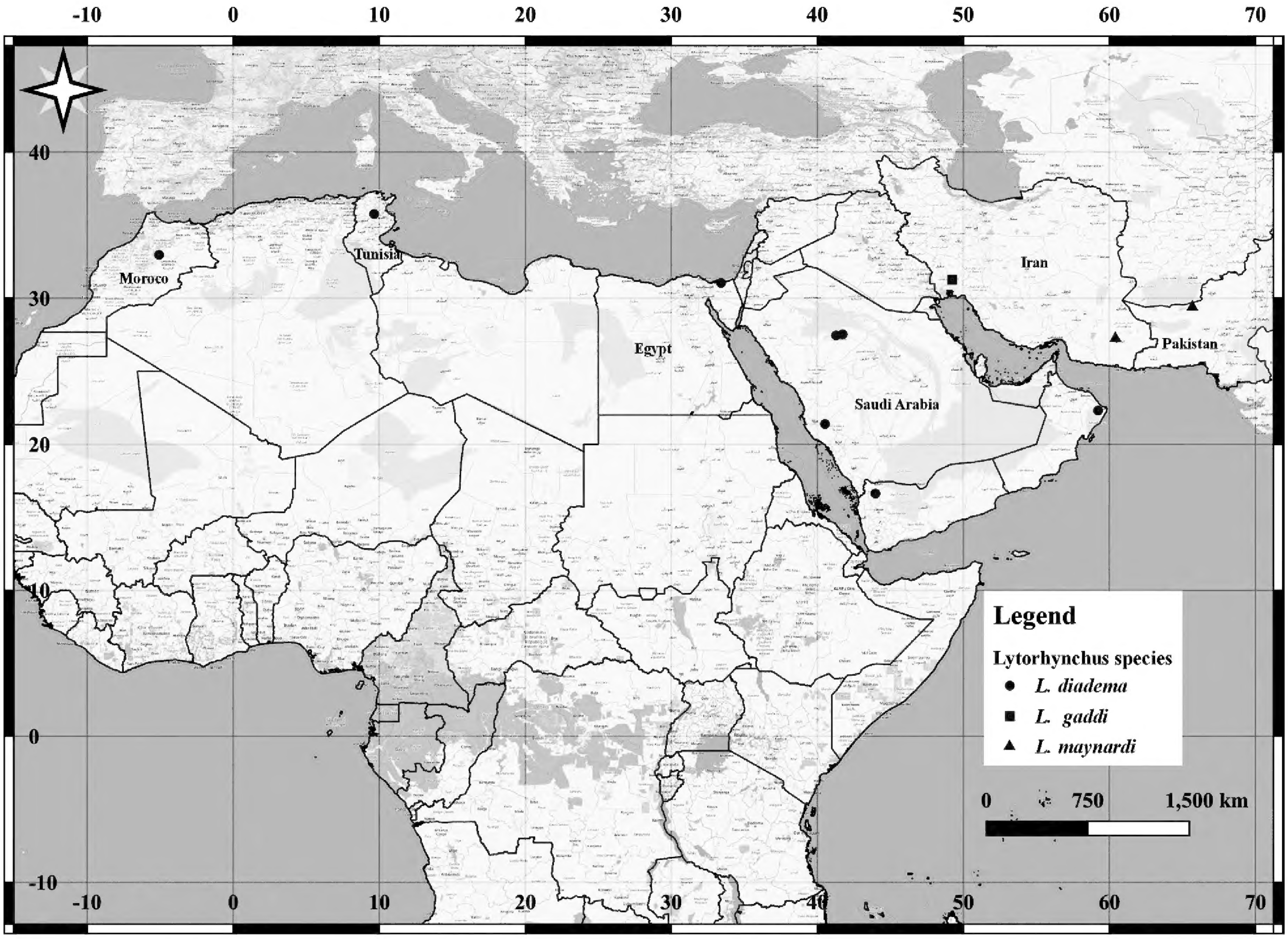

Figure 1. Collection localities of *Lytorhynchus* samples from Saudi Arabia and GenBank sequences previously used in phylogenetic studies (see Table 1 for information about localities).

rules stated in the New York Academy of Sciences (1988) and DNA was extracted from blood samples as described by Alshammari et al. (2015). Partial sequences of 12S and 16S rRNA (lengths of 518–629 and 423 bp for 16S and 12S rRNA, respectively) were amplified by PCR using sequence-specific primers, visualized, and sequenced according to Pook et al. (2009) and Alshammari et al. (2015). The obtained sequences were analyzed and submitted to GenBank (Table 1). Additional sequences of *L. diadema* from Egypt, Tunisia, Morocco, Yemen, and Oman, as well as available data sequences for other species of genus *Lytorhynchus* from Iran and Pakistan (Table 1), were downloaded from GenBank. Additional sequences of other genera were retrieved from GenBank to investigate the monophyly and phylogenetic position of *Lytorhynchus* within Colubrinae. *Coelognathus flavolineatus* (Schlegel, 1837) was used as an outgroup (MG673301 and AY039162).

Phylogenetic analyses

FinchTV 1.4.0, was used to screen and analyze sequences. Sequences were aligned using ClustalW in Mega 6 using the default settings (Tamura et al. 2013). The aligned 12S and 16S sequences were concatenated and combined into a single alignment using the Mesquite v3.2 software (Maddison and Maddison 2018), and the nucleotide composition was calculated. To estimate the sequence divergence for the whole data set, genetic distances were calculated using Mega 6. Phylogenetic analyses were performed on the combined data set (n=15), as well as separate analyses on the individual gene performed to determine the signal in the individual gene. The Maximum-parsimony (MP) and neighbor-joining (NJ) analyses were performed with Paup v4 (Swofford 2001) with heuristic searches using stepwise addition followed by tree bisection reconnection (TBR) branch swapping (Swofford et al. 1996). In all alignments, gaps were treated as missing characters. Confidence within the nodes was evaluated using 1000 bootstrap replicates (Felsenstein 2002) with random addition of taxa. MrModeltest 2.3 (Nylander 2004) was used to select the best-fit models of nucleotide evolution supported by Akaike information criterion (AIC) (Akaike 1973). The geographic structure was inferred using Bayesian inference (BI) implemented with MrBayes 3.1.2 (Ronquist et al. 2012). Analyses were run for one million generations and the output parameters were visualized to determine stationarity and convergence using Tracer 1.4 (Rambaut and Drummond 2007).

Results

Genetic divergence

Across all combined sequences, there were 766 aligned nucleotides. Of these, 620 bases (80.9%) were constant; 138 (18.0%) were variable, and 94 (12.2%) were parsimony informative. Within the 766 bp, 44 polymorphic segregating sites were detected. Divergence among *Lytorhynchus* samples ranged from 0 to 0.04 (Table 2). For the 16S rRNA sequences, there have been 413 aligned nucleotides. Of these, 313 bases (75.7%) were constant; 98 (23.7%) were variable, and 72 (17.4%) were parsimony informative. Within the 413 bp, 62 polymorphic segregating sites were detected. Divergence among *Lytorhynchus* samples ranged from 0 to 0.04 (Suppl. material 1: Table S1). For the 12S rRNA sequences, there have been 352 aligned nucleotides of which 255 (72.4%) were constant, 95 (26.9%) were variable, and 65 (18.4%) were parsimony informative. Within the 413 bp, 30 polymorphic segregating sites were detected. Divergences among *Lytorhynchus* samples ranged from 0 to 0.08 (Suppl. material 1: Table S2).

Phylogenetic analyses

NJ, MP, and BI analyses identified two main clades within *L. diadema* (Fig. 2, Suppl. material 1: Figs S1, S2). The first clade includes all Arabian and African populations and the second clade consisted of *L. gaddi* specimens from Iran. The first clade is further divided into the population of Arabia (including specimens from Yemen, Oman, and Saudi Arabia), and populations from North Africa (composed of specimens from Egypt, Tunisia, and Morocco). The Arabian subclade represented two sister phylogroups; one represents populations within the Eastern and southern parts of Saudi Arabia (Hail and Taif), and Yemen (Jabal Mafluq), whereas a second represents a specimen from Oman.

Discussion

The current study has documented for the first time higher diversification of the 12S rRNA gene in inferring the phylogenetic relationship of *L. diadema* irrespective of the 50% lower polymorphism of the 12S rRNA than 16S rRNA. Thus, more samples and multigene concatenation

Table 2. Uncorrected pairwise distances among *Lytorhynchus* samples based on concatenated mitochondrial 12S rRNA and 16S rRNA sequences. Standard error estimates are shown above the diagonal. SA = Saudi Arabia.

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. ***L. diadema*****, Egypt**	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00
2. ***L. diadema*****, Egypt**	0.00		0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
3. ***L. diadema*****, Morocco**	0.02	0.02		0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
4. ***L. diadema*****, Hail, SA**	0.02	0.02	0.03		0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
5. ***L. diadema*****, Taif, SA**	0.02	0.02	0.03	0.00		0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01
6. ***L. diadema*****, Hail, SA**	0.03	0.03	0.03	0.01	0.01		0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
7. ***L. diadema*****, Hail, SA**	0.03	0.03	0.03	0.01	0.01	0.00		0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
8. ***L. diadema*****, Oman**	0.03	0.03	0.03	0.02	0.02	0.02	0.02		0.01	0.01	0.01	0.01	0.01	0.01	0.01
9. ***L. gaddi***	0.05	0.05	0.05	0.04	0.04	0.04	0.04	0.04		0.01	0.01	0.01	0.01	0.01	0.01
10. ***L. maynardi***	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.05		0.00	0.01	0.01	0.01	0.01
11. ***L. maynardi***	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.05	0.01		0.01	0.01	0.01	0.01
12. ***Rhynchocalamus melanocephalus***	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.08	0.07	0.06	0.06		0.01	0.01	0.01
13. ***Rhynchocalamus satunini***	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.05	0.06	0.04		0.01	0.01
14. ***Boiga_kraepelini***	0.07	0.07	0.08	0.07	0.07	0.07	0.07	0.07	0.08	0.06	0.06	0.06	0.06		0.01
15. ***Coelognathus flavolineatus*** **(outgroup)**	0.07	0.07	0.08	0.08	0.08	0.08	0.08	0.07	0.08	0.07	0.06	0.07	0.08	0.07	

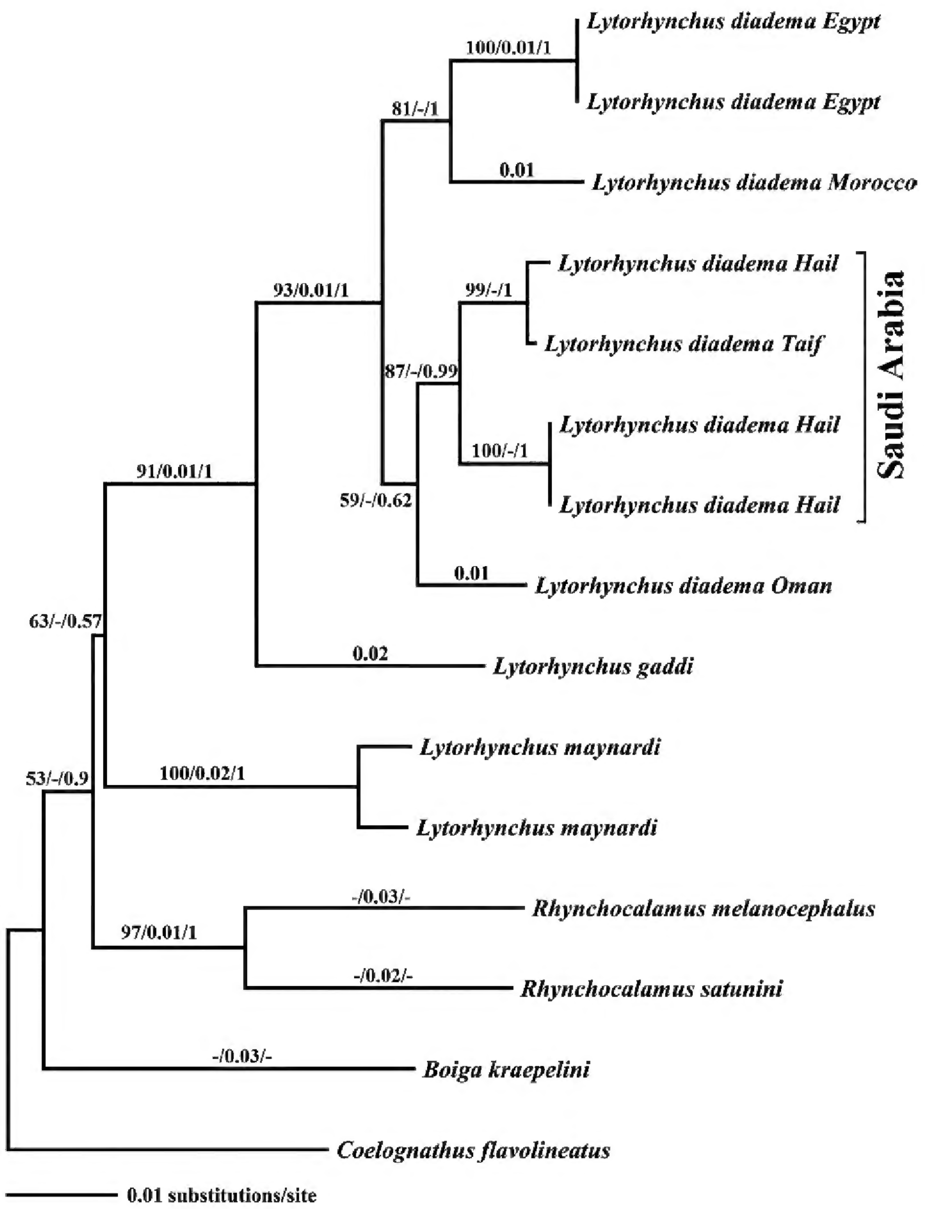

Figure 2. Neighbor-Joining phylogenetic tree of the *Lytorhynchus* species based on the concatenated mitochondrial 12S rRNA and 16S rRNA sequences. Numbers above and below branches indicate MP bootstrap values/NJ distance values/ Bayesian posterior probabilities.

approaches are recommended for more robust, discriminative, and reliable phylogenetics.

The phylogenetic analyses (NJ, MP, and BI) strongly support the monophyly of the genus *Lytorhynchus* (Fig. 2), based on two combined genes representing different species of Colubridae. When analyzed separately, the 12S rRNA gene supports the monophyly of *Lytorhynchus*; however, the16S rRNA gene showed a sister relationship between *Lytorhynchus* and *Rhynchocalamus*. Šmíd et al. (2015) showed that the two genera *Lytorhynchus* and *Rhynchocalamus* shared several morphological characters such as "enlarged wedge-shaped rostral shield and reduced dentition (6–9 and 6–8 maxillary teeth)". Thus, all phylogenetic analyses (MP, NJ, and BI) support the monophyletic status of the genus *Lytorhynchus* from Asia and Africa based on the concatenated analysis and the 12S rRNA gene separately. Previous assemblage studies suggested the monophyly of most members within subfamily Colubrinae based on molecular DNA sequences and morphology (McDowell 1987; Rossman and Eberle 1977; Heise et al. 1995; Kraus and Brown 1998; Vidal et al. 2000; Kelly et al. 2003; Rajabizadeh et al. 2020). Furthermore, Tamar et al. (2016) referred to the monophyly of *Rhynchocalamus*, which had separated from *Lytorhynchus* during the Late Oligocene at ca. 26 Mya. Thus, our molecular data documented a monophyletic relationship for all included *Lytorhynchus* species. However, more comprehensive analyses with additional representative species of this genus should be conducted.

The taxonomic status of *L. gaddi* has been discussed previously (Leviton et al. 1992; Schätti and Gasperetti 1994; Amr and Disi 2011). The divergence between *L. gaddi* and species of genus *Lytorhynchus* ranged from 4–5%, 2–4%, 5–6% for the two concatenated genes, 16S and 12S, respectively (Table 2, Suppl. material 1: Tables S1, S2). *Lytorhynchus gaddi* is distributed in the coastal zones bordering Iran and Oman (Shafiei et al. 2015). It can be distinguished from *L. diadema* by numerous morphologic features as suggested by Leviton and Anderson (1970). Our results revealed that *L. diadema* from Arabia and North Africa nested as a sister group to the specimens of *L. gaddi* from Iran in all phylogenetic analyses, and it was predicted to have diverged from *L. gaddi* (Fig. 2). Also, our result supports the species status of *L. diadema*, *L. maynardi*, and *L. gaddi* and this divergence might be due to vicariant events during the Miocene that might have supported the diversification among the Arabian and Eurasian taxa (Rögl 1999; Harzhauser and Piller 2007; Tamar et al. 2016).

Our phylogenetic results indicate a distinct geographic division between the Arabian populations from Saudi Arabia, Yemen, and Oman and those from North African populations from Egypt, Tunisia, and Morocco, with a genetic divergence of 4–8%. A similar geographical separation based on morphological and molecular data was previously detected by Lawson et al. (2005) among *Lytorhynchus* and other colubrids. Previous studies reported an association between the Red Sea formation and the speciation between Arabian and African lineages in various faunal groups (Sanmartín 2003; Amer and Kumazawa 2005; Derricourt 2005; Tamar et al. 2016; Saleh et al. 2018; Alqahtani and Badry 2020a, b). The diversification of the Arabian species is likely primarily due to the progressive aridification events during the Late Pleistocene and the early Holocene, as suggested by other researchers (Bray and Stokes 2004; Lowe et al. 2014). Moreover, paleoclimatic effects also would have had a marked contribution to the distribution and the speciation of numerous species as reported previously (Lourenço 2020).

In conclusion, this study demonstrated a clear geographic division within the species *L. diadema*, with strong support for a monophyletic relationship, sister to *L. gaddi*. Additional detailed morphological and molecular revisions are required to clarify the relationships between Saudi *L. diadema* and other species of this genus.

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Supplementary material 1

Figures S1, S2; Tables S1, S2

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Explanation note: Figure S1. Neighbor-Joining phylogenies of *Lytorhynchus* spp. DNA sequences fragment of the 16S region from Saudi Arabia. Figure S2. Neighbor-Joining phylogenies of *Lytorhynchus* spp. DNA sequences fragment of the 12S region from Saudi Arabia. Table S1. The uncorrected p distance of the sequence divergence of 16S mtDNA sequences between *Lytorhynchus* samples included in this study. Table S2. The uncorrected p distance of the sequence divergence of 12S mtDNA sequences between *Lytorhynchus* samples included in this study.

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